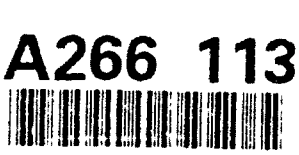

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Abstract

The long-term goal of these studies is to understand how cells of the suprachiasmatic nucleus (SCN) are organized to form a 24-h biological clock and what roles specific neurotransmitters and modulators play in timekeeping and resetting processes. We address these questions by assessing the pattern of spontaneous neuronal activity using extracellular and whole cell patch recording techniques in long-lived SCN brain slices from rat. We have observed that a robust pacemaker persists in the ventrolateral region of microdissected SCN and have begun to define the electrophysiological properties of neurons in this region. Further, we are investigating changing sensitivities of the SCN to resetting by exogenous neurotransmitters, such as glutamate, serotonin and neuropeptide Y, across the circadian cycle. Our findings emphasize the complexity of organization and control of mammalian circadian timing.

The central role of the suprachiasmatic nuclei (SCN) in the mammalian circadian system is well established. An endogenously pacemaking tissue, the SCN exhibit a near 24-h period in intrinsic rhythms of electrical activity and vasopressin secretion (Earnest & Sladek, 1987; Gillette & Reppert, 1987; Prosser & Gillette, 1989). Outputs from this central pacemaker supply a time-base for circadian rhythms in cellular, tissue and organismic functions. Behavioral circadian rhythms are reset differentially over the 24-h circadian cycle by variables that include environmental lighting (DeCoursey, 1984; Boulos & Rusak, 1982) and activity state (Mrosovsky & Salmon, 1987). These phase-resetting stimuli must affect the SCN through input pathways, such as those from the retina (Moore & Lenn, 1972), intergeniculate leaflet (Swanson *et al.*1974; Card & Moore, 1982) or dorsal raphe (Aghajanian *et al.*1969; Moore *et al.*1978). However, little is known about the way in which the cellular components of the SCN are organized to carry out time-keeping or to analyze phase-resetting information. We seek to determine 1) the functional organization of the SCN by electrophysiological analyses of regional distribution of pacemaking properties and neuronal characteristics as well as 2) the circadian nature of SCN pacemaker regulation by neurotransmitters and modulators.

We use the hypothalamic brain slice (Hatton *et al.*1980) to study the functional organization of the SCN directly. Slices are prepared from Long-Evans rats, raised to 5-10 weeks of age on a 12L:12D lighting cycle in our inbred colony. Our previous work has established that circadian pacemaking and resetting properties are endogenous to the SCN in slice and can be analyzed *in vitro* (for review see Gillette, 1991). The circadian

rhythm of SCN electrical activity was recorded extracellularly in intact and microdissected slices of rat hypothalamus for 1-3 days after slice preparation. Persistence of a rhythm in microdissected subregions was examined. Whole cell patch recording in slice (Blanton *et al.*1989) of single SCN neurons was performed over the circadian cycle to assess the range of electrophysiological features of SCN neurons together with diurnal changes in electrical properties. Neurotransmitters and neuromodulators were applied focally with micropipette to their SCN projection sites; effects on the phase of the electrical activity rhythm were determined from the behavior of the ensemble of single units. Additionally, the levels of glutamic acid decarboxylase (GAD, the biosynthetic enzyme for the inhibitory neurotransmitter GABA) in SCN micropunched from brain slices were assessed over the circadian cycle by Western blotting. In experiments aimed at understanding regulation by retinohypothalamic afferents, Dr. Michael Rea's lab at the USAF-SAM has examined several parameters after stimulation of the optic nerve: the release of excitatory amino acids, field potential activity and sensitivity to pharmacological blockade of SCN *in vitro*; these results will be discussed in Dr. Rea's paper.

Hypotheses tested in this study include: 1) pacemaking properties are distributed throughout the SCN; 2) the neurons of the SCN are homogeneous with respect to their electrical and pacemaking properties; 3) neuromodulators from inputs implicated in phase-shifts of behavior by dark pulses (serotonin from the raphe, neuropeptide Y from the intergeniculate leaflet) are effective phase-shifting agents for SCN during the circadian day; 4) GAD levels are constant over the circadian cycle; and 5) light information

carried by the retinohypothalamic tract affects the SCN via excitatory amino acids (*viz.*, glutamate). Our progress toward evaluating these hypotheses is presented here.

Regarding the first hypothesis, cellular organization of the SCN pacemaker was examined by determining whether regional variation in the circadian oscillation in ensemble neuronal activity was expressed in the intact SCN brain slice. *Post hoc* analysis of the pattern of activity recorded on day 2 *in vitro* revealed that, indeed, both the VL and DM subregions exhibit pronounced activity peaks (Tcheng *et al.*1989). These were not apparently altered by bisecting the brain slice at the base of the third ventricle, which severs connections between the bilaterally paired SCN. In other words, each SCN appears to be an autonomous pacemaker.

The rat SCN has two natural anatomical subdivisions, 1) the *ventrolateral SCN* (VL-SCN), site of the relatively larger neurons (mean diameter 9.6$\pm$1.5 μM), including those containing vasoactive intestinal peptide (VIP) upon which afferents from the retina, intergeniculate leaflet and dorsal raphe form synapses (Van Den Pol & Tsujimoto, 1985; Guy *et al.*1987), and 2) the *dorsomedial SCN* (DM-SCN), which is composed of relatively smaller vasopressin-containing neurons (mean diameter 7.8$\pm$0.9 μm) which give rise to numerous efferent fibers (van den Pol, 1980). We hemisected each SCN so as to separate the VL-SCN from the DM-SCN in order to determine the pacemaking ability of each region. When activity in a single intact SCN was monitored continuously for 32 h, the characteristic sinusoidal circadian pattern in firing frequency (Gillette & Prosser, 1988) was

observed with high amplitude peaks at 24-h intervals (Gillette *et al.*1992). Surprisingly, hemisection did not effect this pattern in the VL-SCN, whereas amplitude and shape of the neuronal activity rhythm appeared altered in the DM-SCN after surgical isolation (Tcheng & Gillette, 1990; Gillette *et al.*1992). These preliminary results are consistent with the alternative to the hypothesis tested, namely, that there may be localization of pacemaking function within the SCN.

In order to address the second hypothesis, the issue of neuronal heterogeneity at the cellular level, we have begun to examine individual neurons of the SCN using the whole cell patch recording technique (Blanton *et al.*1989) in the rat brain slice. Initial efforts were concentrated in the VL-SCN, although we have recently begun to extend our observations to DM-SCN. By using this approach, we have found the SCN to be composed of a variety of electrophysiologically distinct cell types (Gallman *et al.*1991; Gallman & Gillette, Submitted 1993). Our observations challenge the hypothesis, which had been suggested by early workers in the field (Wheal & Thompson, 1984), that the SCN is electrically homogeneous. Furthermore, preliminary findings concerning DM-SCN neurons do not contradict the alternative hypothesis, that there *are* regional differences in properties of neurons of the VL- and DM-SCN.

The third hypothesis concerns the potential role of serotonin (5HT) and neuropeptide Y (NPY) in mediating phase-shifts induced by behavioral arousal and dark pulses, respectively. This was tested by applying a 30 *fl* droplet of either 10^{-6} M 5-HT or

NPY, to the VL-SCN in brain slice. Effects upon the rhythms of neuronal activity of 5 min microdrop applications at various points in the circadian cycle were assessed over 1-2 days post-treatment. These experiments were designed to evaluate permanence, receptor specificity, and dose-dependency of phase changes compared with controls treated with microdrops of medium. SCN sensitivity to 5-HT was restricted to subjective day, between circadian time 2-11 (CT 2-11), with peak phase-advance occurring at CT 7 (Fig. 1). Both 5-carboxyamidotryptamine (5-CT), a $5HT_1$ receptor agonist, and 8-hydroxy-2-*n*-propylamino)-tetralin (8-OH DPAT), an agonist specific for the $5\text{-}HT_{1A}$ receptor subtype, also induce large advances in the peak of the electrical activity rhythm in SCN *in vitro*; these treatments were without effect during subjective night (Medanic & Gillette, 1992a). Thus, the regulatory effect of 5-HT on the neurons of the VL-SCN appears to be mediated through a $5\text{-}HT_{1A}$ receptor subtype. Interestingly, the SCN in slice shows both late day and late night windows of sensitivity to NPY-induced phase resetting (Medanic & Gillette, ~~1993~~ submitted). The daytime period of sensitivity to NPY is distinct from, but overlaps with the latter portion of 5-HT sensitivity (Medanic & Gillette, 1992b). These results are consistent with roles for serotonin and/or neuropeptide Y in nonphotic (Mrosovsky & Salmon, 1987) and/or dark pulse (Albers & Ferris, 1984; Boulos & Rusak, 1982; Ellis *et al.*1982) entrainment processes.

Next we examined the hypothesis that levels of glutamic acid decarboxylase (GAD) remain constant over the circadian cycle. GAD is the biosynthetic enzyme for the most abundant inhibitory neurotransmitter in the SCN, γ-aminobutyric acid (GABA). Between

50-100% of SCN neurons are GAD positive (van den Pol and Moore, personal comm.) GABA-ergic neurons, those containing GAD, are distributed throughout the nucleus (Van den Pol & Tsujimoto, 1985), and GABA administration inhibits 65% of SCN neurons (Liou *et al.*1990). The amplitude of the daily oscillation of mean spontaneous firing frequency for rat SCN neurons shows an excursion between 8 Hz at midday and 2 Hz at midnight. This may be regulated, at least in part, by changing GABA levels, which in turn would be controlled by changing GAD levels and/or GAD activity over the course of the circadian cycle. To evaluate this issue, SCN from brain slices maintained *in vitro* were rapidly frozen on dry ice and the SCN removed by micropunch. Western blot analysis of the two major GAD isozymes, GAD_{65} and GAD_{67}, demonstrated that both are present in SCN at the four 6-h intervals examined, and that the levels undergo a significant circadian variation (Richard *et al.*1991). Whether circadian modulation of GAD activity also affects GABA biosynthesis in the SCN is currently under investigation.

The fifth hypothesis tested in this study regards the potential role of excitatory amino acids in mediating the effects of light at night in this system. These experiments were addressed primarily by Dr. Michael Rea's lab at the USAF-SAM, as discussed in his paper in this volume. Our laboratory has begun to evaluate the effect on the circadian rhythm of ensemble neuronal activity of the excitatory amino acid glutamate (GLU). GLU (at 10^{-5}M in a 1 μl droplet of medium) was applied for 10 min to one SCN *in vitro*. Preliminary results suggest that this brief GLU application can induce both phase delays and advances at night, and strengthens the possibility that GLU may mediate the phase-

shifting effects of light on the SCN pacemaker.

With the finding that pacemaking properties reside in the VL-SCN (at least), we can proceed to focus upon the electrophysiological properties of this region. Differences between VL- and DM-SCN will be interesting to document. Circadian periods of sensitivity to 5-HT, NPY and GLU, tentatively described in this report, differ from those we have described for cAMP (Prosser & Gillette, 1989), cGMP (Prosser *et al.*1989) and melatonin (McArthur *et al.*1991), emphasizing the complexity of SCN regulatory processes. Because the SCN integrate most circadian behaviors and metabolic fluxes, this study has basic relevance to understanding circadian dysfunction induced by transmeridian travel and sustained, irregular work schedules, with application to improving human performance under conditions that induce circadian desynchronization.

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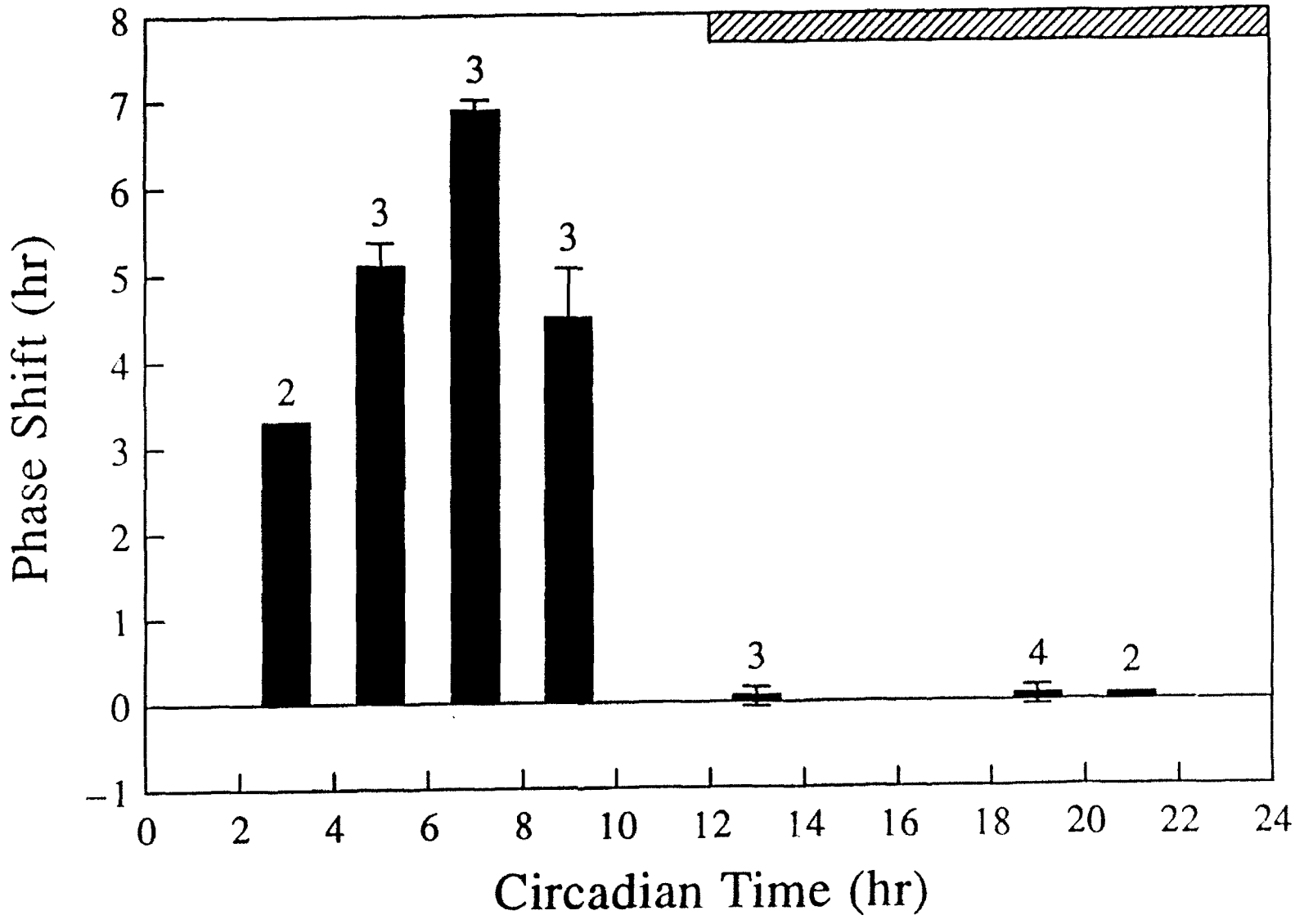
Figure Legend

Figure 1. Phase response curve relating the time of serotonin treatment to the time of appearance of the peak in ensemble neuronal firing rate in the next circadian cycle. For treatments at CT 7, the time of maximal phase advance, activity was monitored for two days post-treatment. The peak on day 3 appeared at CT 0, 24-hr after that on day 2, demonstrating that the phase-shift measured on day 2 is complete. The hatched horizontal bar represents subjective night. (After Medanic and Gillette, 1992a.)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